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Antagonism by (R)- and (S)-trihexyphenidyl of muscarinic stimulation of adenylyl cyclase in rat olfactory bulb and inhibition in striatum and heart

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1 Activation of muscarinic receptors in rat olfactory bulb stimulates adenylyl cyclase activity. This response was competitively antagonized by the (R)- and (S)-enantiomers of trihexyphenidyl with pA_2 values of 8.84 and 6.09, respectively.

2 Similarly, in rat striatal homogenates, muscarinic inhibition of adenylyl cyclase activity was antagonized by the (R)- and (S)-enantiomers with pA_2 values of 8.75 and 6.12, respectively.

3 In contrast, in rat myocardium the muscarinic inhibition of the adenosine 3':5'-cyclic monophosphate (cyclic AMP) formation was more weakly antagonized by trihexyphenidyl, with a particularly marked loss (15 fold) in activity of the (R)-enantiomer. The (R)- and (S)-enantiomers had pA_2 values of 7.64 and 5.72, respectively.

4 Each muscarinic response was completely antagonized by increasing concentrations of (R)-trihexyphenidyl with a Hill coefficient not significantly different from unity.

5 The present study shows that the muscarinic receptors coupled to stimulation of adenylyl cyclase in the olfactory bulb display high stereoselectivity for the enantiomers of trihexyphenidyl. The affinities of these receptors for the antagonists are similar to those shown by the striatal receptors. This finding supports the hypothesis that both the muscarinic stimulation of adenylyl cyclase in the olfactory bulb and the muscarinic inhibition of the enzyme in striatum are mediated by activation of a receptor subtype pharmacologically equivalent to the m4 gene product. On the other hand, the weaker affinities and the lower stereoselectivity for the trihexyphenidyl enantiomers exhibited by the muscarinic inhibition of adenylyl cyclase in the heart are consistent with the involvement of M_2 receptors in this response.

Keywords: Trihexyphenidyl enantiomers; muscarinic receptor subtypes; cyclic AMP; adenylyl cyclase; rat heart, rat striatum; rat olfactory bulb

Introduction

Activation of muscarinic cholinoceptor has been reported to inhibit adenylyl cyclase activity in different rat brain areas, such as corpus striatum (Olianas *et al.*, 1983), cerebral cortex (McKinney *et al.*, 1991) and hippocampus (Vickroy & Cadman, 1989). However, in rat olfactory bulb, muscarinic receptor agonists cause stimulation, rather than inhibition, of basal adenylyl cyclase activity (Onali & Olianas, 1990). The muscarinic stimulation does not require Ca^{2+} (Olianas & Onali, 1990), is prevented by *in vivo* treatment with pertussis toxin (Olianas & Onali, 1992) and is enhanced by concurrent activation of the enzyme by G_s-coupled neurotransmitter receptors (Olianas & Onali, 1993a). Unlike the muscarinic stimulation of adenosine 3':5'-cyclic monophosphate (cyclic AMP) accumulation described in cells either transfected with the muscarinic m1 receptor gene (Felder *et al.*, 1989) or naturally expressing the M_3 receptor subtype (Baumgold *et al.*, 1992), the muscarinic stimulation of adenylyl cyclase in rat olfactory bulb occurs independently of activation of phosphoinositide hydrolysis (Olianas & Onali, 1993b) and may result from a direct coupling of the receptors to the catalyst through guanosine-5'-triphosphate (GTP) binding regulatory proteins of the G_o/G_i family (Olianas & Onali, 1993a).

The pharmacological properties of the muscarinic receptors involved in this unique response appear different from those typically displayed by the M_1, M_2 and M_3 receptor subtypes. In fact, the muscarinic stimulation of adenylyl cyclase activity of rat olfactory bulb is highly sensitive to either classical M_2 antagonists, such as methoctramine and AF-DX 116, or M_3 antagonists, such as hexahydrosiladiphenidol and *p*-fluorohexahydrosiladiphenidol, and is moderately sensitive to the M_1 antagonists, pirenzepine (Olianas & Onali, 1991). The data so far accumulated have led to the hypothesis that the predominant receptor involved may be pharmacologically equivalent to the m4 gene product (M_4) (Olianas & Onali, 1991). However, because selective M_4 antagonists are not currently available, the characterization of the receptor subtype(s) involved requires the comparison amongst the increasing number of drugs which can discriminate between subtypes and with other responses with well characterized pharmacological profiles. Muscarinic receptor subtypes can be differentiated on the basis of their stereoselective interaction with chiral antagonists (Waelbroeck *et al.*, 1989). For the antimuscarinic drug, trihexyphenidyl, the ratio between the potencies of the (R)- and (S)-enantiomer, the eudismic ratio (Lehmann, 1986), has been found to differ markedly when the compounds were tested as antagonists of functional responses mediated by M_1, M_2 and M_3 receptors (Lambrecht *et al.*, 1988). Moreover, in radioligand binding studies employing the five cloned muscarinic receptors subtypes, (R)-trihexyphenidyl has been found to display high affinity for the m1 and m4 and low affinity for the m2 and m5 subtypes (Dorje *et al.*, 1991). In the present study we have investigated the sensitivity of the muscarinic stimulation of adenylyl cyclase in rat olfactory bulb to the antagonistic activity of the trihexyphenidyl enantiomers. For comparison, the compounds were also tested on the muscarinic inhibition of adenylyl cyclase in rat striatum and myocardium.

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Methods

Tissue homogenization

Hearts, olfactory bulbs and striata were obtained from male Sprague-Dawley rats (200–350 g) killed by decapitation. Crude membrane preparations were obtained from each tissue according to the following procedures.

Hearts were perfused *in situ* with ice-cold saline solution, excised and minced with a razor blade. The tissue was homogenized in 10 volumes (v/w) of an ice-cold hypotonic homogenization buffer containing 10 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES)/NaOH, 1 mM ethylene glycol *bis* (β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA), 1 mM $MgCl_2$ and 1 mM dithiothreitol (DTT) (pH 7.4) in a Polytron. The homogenate was filtered through two layers of gauze and centrifuged at 1,000 *g* for 10 min at 4°C. The supernatant was discarded and the pellet resuspended in 10 volumes of homogenization buffer. The tissue suspension was centrifuged at 28,000 *g* for 10 min at 4°C and the final pellet was resuspended in the same buffer at a protein concentration of 5.0 to 6.5 mg ml^{-1}.

Olfactory bulbs were rapidly dissected from the brain, stripped of meninges and homogenized by hand in 10 volumes of ice-cold hypotonic homogenization buffer. The homogenate was diluted six fold and centrifuged at 27,000 *g* for 20 min at 4°C. The pellet was resuspended in the same buffer and centrifuged as above. The final pellet was resuspended to a protein concentration of 1.0 to 1.5 mg ml^{-1}, incubated for 5 to 10 min in an ice-bath and then used for adenylyl cyclase assay. Striata were homogenized in 10 volumes of an ice-cold isotonic buffer containing 0.32 M sucrose, 10 mM HEPES/NaOH, 1 mM EGTA and 1 mM DTT (pH 7.4). The homogenate was diluted twofold and centrifuged at 1,000 *g* for 10 min at 4°C. The supernatant was aspirated and centrifuged at 11,000 *g* for 20 min at 4°C. The pellet was resuspended in 40 volumes of ice-cold hypotonic buffer containing all the constituents of the homogenization buffer except sucrose. The tissues was then centrifuged at 27,000 *g* for 20 min at 4°C and the final pellet was resuspended in hypotonic buffer to a protein concentration of 1.2 to 1.6 mg ml^{-1}. For each tissue, at lest three separate membrane preparations were tested.

Adenylyl cyclase activity

The enzyme activity was assayed in a 100 μl reaction mixture containing 50 mM HEPES/NaOH buffer (pH 7.4), 2.3 mM $MgCl_2$, 1.3 mM DTT, 0.3 mM EGTA 0.2 mM [α-^{32}P]adenosine-5'-triphosphate (ATP) (45–60 c.p.m. $pmol^{-1}$), 1 mM [^{3}H]-cyclic AMP (80 c.p.m. $nmol^{-1}$), 1 mM 3-isobutyl-1-methylxanthine, 5 mM phosphocreatine, 50 u ml^{-1} of creatine kinase, 100 μM GTP, 50 μg of bovine serum albumin, 10 μg of bacitracin, 10 kallikrein inhibitor units of aprotinin and 10 μM physostigmine. When the enzyme activity was assayed in the myocardium and striatum, the concentration of [α-^{32}P]ATP was 0.05 mM and 10 μM forskolin, a direct stimulator of adenylyl cyclase, was added to the reaction mixture to amplify the muscarinic inhibition. The incubation was started by adding the tissue preparation (40–70 μg of protein) and was carried out at 30°C for 10 min. [^{32}P]-cyclic AMP was isolated as described by Salomon *et al.* (1974).

Protein content was determined by the method of Bradford (1976), with bovine serum albumin used as a standard.

Statistical analysis

Results are reported as mean ± standard error of the mean (s.e.mean). Agonist concentration-response curves were analysed by a least squares curve fitting computer programme (Graph-Pad, ISI, Philadelphia, PA, U.S.A.). The antagonist effects were examined according to Schild analysis (Schild, 1947) and the potency of each antagonist was determined from the ratios between the EC_{50} values of the agonist estimated in the absence and in the presence of multiple concentrations of the antagonist. The pA_2 values were determined from the x intercepts and calculated by least squares regression analysis of the Schild plots, where the log of the dose ratios (DR) − 1 is plotted as a function of the antagonist concentration. The (**R**)-enantiomer was also tested for its ability to reverse completely the agonist response. In these experiments the effects of multiple concentrations of the antagonist on the response elicited by a fixed concentration of the agonist were determined. The data were analysed as competition curves by nonlinear regression analysis for models of one or two noninteracting sites. The antagonist inhibitory constant (K_i) was calculated according to the equation:

$$K_i = IC_{50}/1 + (A/EC_{50}) \quad (1)$$

where IC_{50} is the concentration of the antagonist producing half-maximal inhibition; A is the concentration of the agonist and EC_{50} is the agonist concentration producing half-maximal effect. The goodness of fit of the data to a one- versus a two site-model was evaluated by the *F* test of the sum of squares of residuals from both fittings considering a level of significance of $P<0.05$. For the easier comparison with the pA_2 values, K_i values were converted to the logarithmic form (pK_i). Statistical significance of the difference between means was determined by Student's *t* test.

Drugs

[α^{32}P]-ATP (30–40 Ci $mmol^{-1}$) and [2,8-^{3}H]-cyclic AMP (25 Ci $mmol^{-1}$) were purchased from Du Pont de Nemours (Bad Homburg, Germany). Forskolin was from Calbiochem (La Jolla, CA, U.S.A.). The enantiomers of trihexyphenidyl were synthesized in the laboratory of A.J.A. The absolute configurations of the (−)- and (+)-enantiomers are (**R**) and (**S**), respectively (Schjelderup *et al.*, 1987). Based on optical activity measurements, the enantiomeric excesses were estimated to be: (**R**)-(−)-trihexyphenidyl 99%, (**S**)-(+)-trihexyphenidyl 98%. Both enantiomers were in the hydrochloride form and were dissolved in distilled water. Physostigmine sulphate, acetylcholine hydrochloride (ACh) and the other compounds used for the adenylyl cyclase assay were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Results

Schild analysis

In rat olfactory bulb, ACh caused a concentration-dependent stimulation of adenylyl cyclase activity with an EC_{50} value of 0.370 ± 0.07 μM and a maximal effect corresponding to a 50.3 ± 6.1% increase of basal enzyme activity ($n = 12$, $P<0.001$). The slope value of the curve was 0.921 ± 0.06 and did not differ significantly from unity. In the presence of increasing concentrations of (**R**)-trihexyphenidyl the ACh response curve was progressively shifted to the right (Figure 1a). A Schild plot of (**R**)-trihexyphenidyl antagonism yielded a pA_2 value of 8.84 and a slope value of 0.908 (Table 1). (**S**)-Trihexyphenidyl shifted the ACh concentration-response curves only at concentrations or equal to higher than 1 μM (Figure 1b). The corresponding Schild plot yielded a straight line and a pA_2 value of 6.09.

In rat striatal membranes ACh maximally inhibited adenylyl cyclase activity by 23.5 ± 1.5% (*P* 0.001) with an EC_{50} value of 1.49 ± 0.12 μM ($n = 9$). (**R**)-trihexyphenidyl antagonized the ACh-induced inhibition of adenylyl cyclase in a concentration-dependent manner with a pA_2 value of 8.75 (Figure 2a; Table 1). Also for this muscarinic response, the (**S**)-enantiomer (Figure 2b) behaved as a weaker antagonist, displaying a pA_2 value of 6.12.

In the heart, ACh produced a concentration-dependent

inhibition of adenylyl cyclase with an EC_{50} value of $0.80 \pm 0.04\,\mu M$ and a maximal effect corresponding to a $45.6 \pm 5.5\%$ reduction of control enzyme activity ($n = 10$, $P < 0.001$). The (R)- and (S)-enantiomers counteracted the response to ACh with pA_2 values of 7.64 and 5.72, respectively (Figure 3).

The eudismic ratios for the antagonism of the muscarinic responses in olfactory bulb, striatum and heart were 562, 426 and 83, respectively.

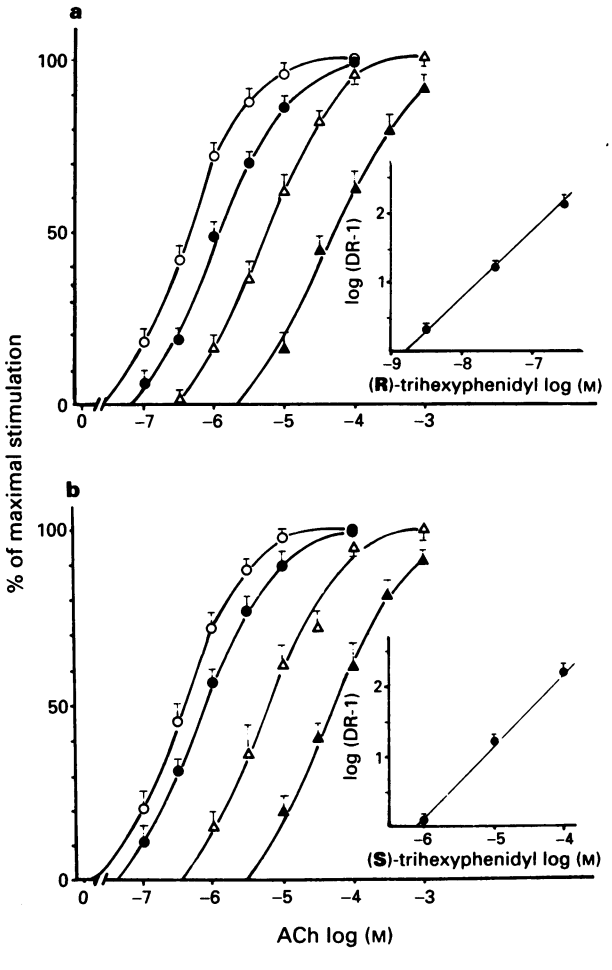

Figure 1 Antagonism of acetylcholine (ACh) stimulation of adenylyl cyclase activity of olfactory bulb by (R)- and (S)-trihexyphenidyl. (a) The enzyme activity was assayed at the indicated concentrations of ACh in the absence (○) and in the presence of 3 nM (●), 30 nM (△) and 300 nM (▲) (R)-trihexyphenidyl. Inset: Schild plot of the antagonism. (b) The enzyme activity was assayed in the absence (○) and in the presence of 1 μM (●), 10 μM (△) and 100 μM (▲) (S)-trihexyphenidyl. Inset: Schild plot of the antagonism. Data are the mean ± s.e.mean of three experiments for each antagonist. Enzyme activities (expressed as pmol cyclic AMP min^{-1} mg^{-1} protein ± s.e.mean) were: (a) basal 115.3 ± 2.7, ACh (100 μM) 169.2 ± 3.4 ($P < 0.01$); (b) basal 110.3 ± 1.8, ACh (100 μM) 161.9 ± 2.8 ($P < 0.01$).

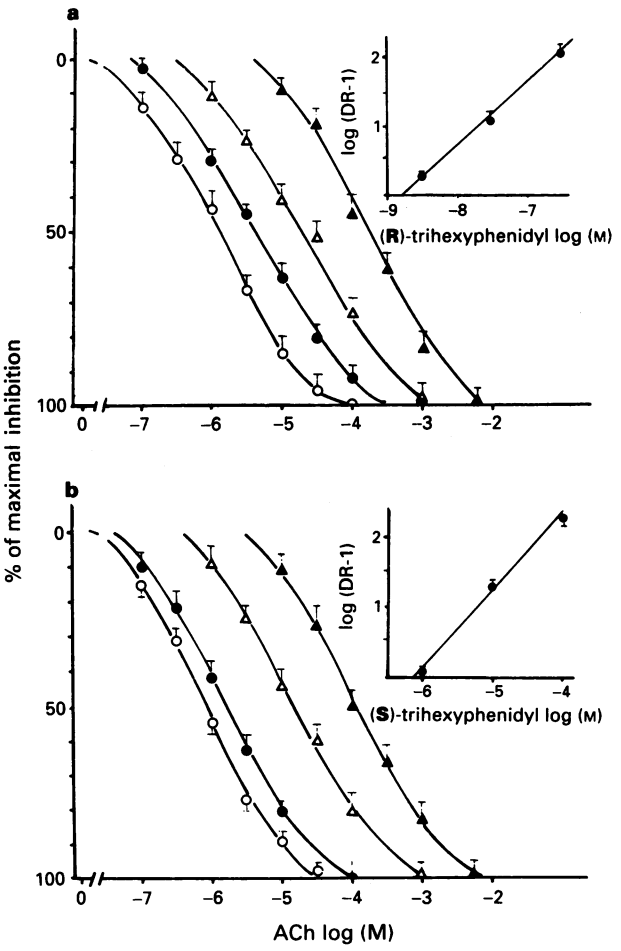

Figure 2 Antagonism of acetylcholine (ACh) inhibition of adenylyl cyclase activity of rat striatum by (R)- and (S)-trihexyphenidyl. (a) The enzyme activity was assayed at the indicated concentrations of ACh in the absence (○) and in the presence of 3 nM (●), 30 nM (△) and 300 nM (▲) (R)-trihexyphenidyl. Inset: Schild plot of the antagonism. (b) The enzyme activity was assayed in the absence (○) and in the presence of 1 μM (●), 10 μM (△) and 100 μM (▲) (S)-trihexyphenidyl. Inset: Schild plot of the antagonism. Data are the mean ± s.e.mean of three experiments for each enantiomer. Enzyme activities (expressed as nmol cyclic AMP min^{-1} mg^{-1} protein ± s.e.mean) were: (a) control, 2.96 ± 0.03, ACh (100 μM) 2.28 ± 0.04 ($P < 0.01$); (b) control 2.89 ± 0.02, ACh (100 μM) 2.21 ± 0.03 ($P < 0.01$).

Inhibition curves

Increasing concentrations of (R)-trihexyphenidyl completely antagonized both the stimulatory and inhibitory effects of ACh (Figure 4). The competition curves were monophasic with Hill coefficients not significantly different from unity (Table 2). Moreover, in each tissue no significant difference ($P > 0.05$) was found by fitting the data to either a two-sites or a one-site competition model. The apparent pK_i values of

Table 1 Affinity constants and eudismic ratios (R/S) of the enantiomers of trihexyphenidyl at muscarinic receptors coupled to stimulation of adenylyl cyclase activity in rat olfactory bulb and to inhibition of the enzyme in striatum and heart

	(R)-trihexyphenidyl		*(S)-trihexyphenidyl*		
Tissue	pA_2	slope	pA_2	slope	(R/S)
Olfactory bulb	8.84 ± 0.02	0.908 ± 0.07	6.09 ± 0.03	1.072 ± 0.04	562
Striatum	8.75 ± 0.05	0.910 ± 0.08	6.12 ± 0.07	1.068 ± 0.06	426
Heart	7.64 ± 0.03	1.050 ± 0.09	5.72 ± 0.05	1.098 ± 0.10	83

Data were obtained from the experiments shown in Figures 1–3. Each value is the mean ± s.e.mean of three experiments. Slope values of Schild plot regression lines were not significantly different from unity ($P > 0.05$). The eudismic ratios were calculated from the antilog of the difference between the pA_2 values of the (R)- and (S)-enantiomers.

(R)-trihexyphenidyl obtained from the competition curves (Table 2) were in good agreement with the corresponding pA_2 values.

Discussion

The present study shows that the stereoisomers of trihexyphenidyl are effective antagonists at muscarinic receptors coupled to either stimulation or inhibition of adenylyl cyclase. For each response examined, the antagonism appears to be competitive, since it is completely surmountable over a wide range of concentrations and is associated with Schild slopes not significantly different from unity. In agreement with previous observations (Barlow, 1971; Lambrecht *et al.*, 1988) the (R)-enantiomer is considerably (80–560 fold) more potent than the (S)-enantiomer. The affinity of the (R)-enantiomer for the muscarinic receptors of the olfactory bulb coupled to stimulation of adenylyl cyclase is similar to that estimated for the striatal receptors linked to inhibition of the enzyme. In terms of absolute values, the potencies of (R)-trihexyphenidyl in antagonizing the ACh effects in the olfactory bulb and striatum correlate well with the reported affinities of the compound for the cloned human m4 and m1 receptor subtypes and for the striatal M_4 sites (Dorje *et al.*, 1991; Waelbroeck *et al.*, 1992). The potency of the (R)-

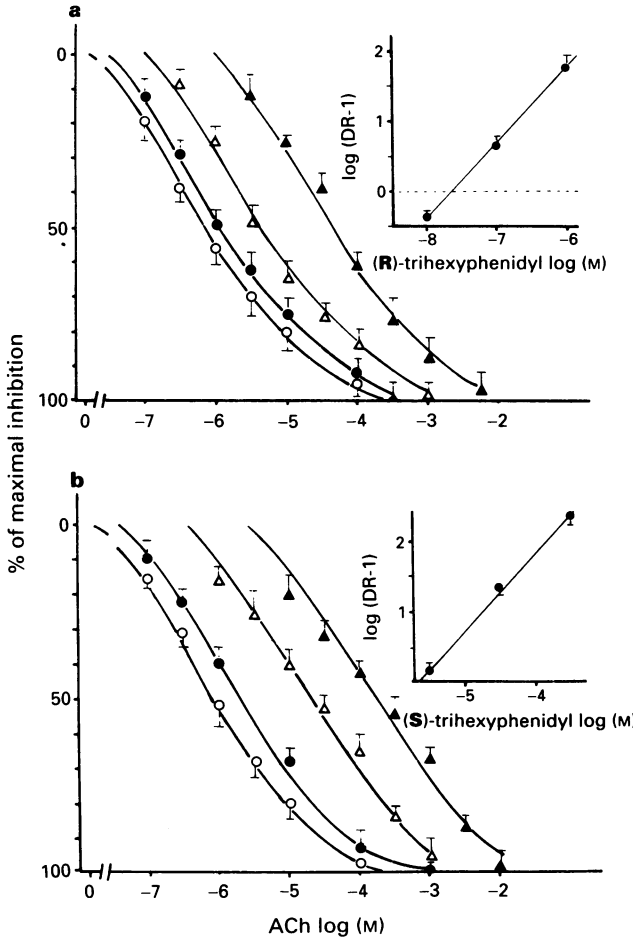

Figure 3 Antagonism of acetylcholine (ACh) inhibition of adenylyl cyclase activity of rat heart by (R)- and (S)-trihexyphenidyl. (a) The enzyme activity was assayed at the indicated concentrations of ACh in the absence (○) and in the presence of 10 nM (●), 100 nM (△) and 1000 nM (▲) (R)-trihexyphenidyl. Inset: Schild plot of the antagonism. (b) The enzyme activity was assayed in the absence (○) and in the presence of 3 μM (●), 30 μM (△) and 300 μM (▲) (S)-trihexyphenidyl. Inset: Schild plot of the antagonism. Data are the mean ± s.e.mean of three experiments for each antagonist. Enzyme activities (expressed as pmol cyclic AMP min^{-1} mg^{-1} protein ± s.e.mean) were: (a) control 70.2 ± 3.5, ACh (300 μM) 38.9 ± 2.3 ($P<0.001$); (b) control 65.9 ± 1.9, ACh (300 μM) 35.2 ± 1.5 ($P<0.001$).

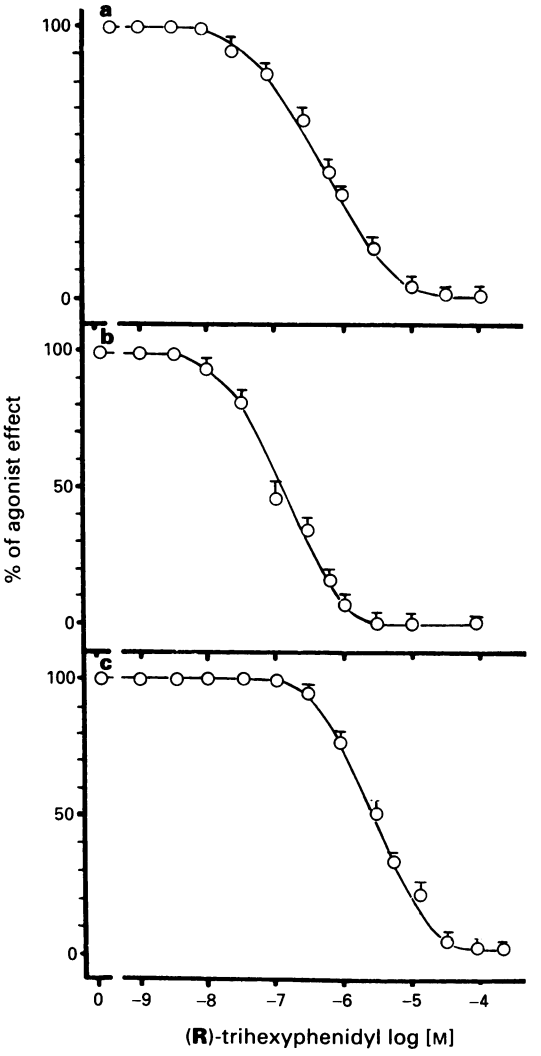

Figure 4 Concentration-dependent inhibition by (R)-trihexyphenidyl of the acetylcholine (ACh)-induced stimulation of adenylyl cyclase of rat olfactory bulb (a) and of the ACh-induced inhibition of the enzyme in rat striatum (b) and heart (c). In each tissue the enzyme activity was assayed as described under Methods at the indicated concentrations of the antagonist in the absence and in the presence of 100 μM ACh. Data are expressed as percentage of the maximal effect elicited by ACh and represent the mean ± s.e.mean of three experiments for each tissue. At the concentrations tested, (R)-trihexyphenidyl failed to affect the enzyme activity *per se*.

Table 2 Inhibition by (R)-trihexyphenidyl of acetylcholine effects on adenylyl cyclase in olfactory bulb, striatum and heart of rat

Tissue	pK_i	*Hill coefficient*
Olfactory bulb	8.77 ± 0.03	0.990 ± 0.03
Striatum	8.76 ± 0.04	1.040 ± 0.02
Heart	7.58 ± 0.02	1.100 ± 0.05

Data were obtained from the experiments shown in Figure 4. Each value is the mean ± s.e.mean of three experiments. pK_i values were calculated according to equation 1 described under Methods. Hill coefficients were not significantly different from unity ($P>0.05$).

enantiomer in antagonizing the ACh inhibition of adenylyl cylcase of rat myocardium is between 10 and 20 fold lower than that observed in the olfactory bulb and striatum and is quite close to its affinity for the cardiac M_2 sites (Waelbroeck *et al.*, 1992). Moreover, the 13–15 fold difference between the potencies in striatum and olfactory bulb and that in myocardium agrees with the selectivity of (R)-trihexyphenidyl for the M_1–M_4 sites versus the M_2 site observed previously (Waelbroeck *et al.*, 1992).

Unlike the (R)-enantiomer, (S)-trihexyphenidyl discriminates poorly between the muscarinic receptors coupled to adenylyl cyclase in the different tissues. Thus, the pA_2 values are only two fold higher in olfactory bulb and striatum than in myocardium. This finding is generally in agreement with the data obtained in functional and radioligand binding studies, in which (S)-trihexyphenidyl was found to possess almost equal affinities for the different muscarinic receptor subtypes (Lambrecht *et al.*, 1988; Dorje *et al.*, 1991; Waelbroeck *et al.*, 1992).

The eudismic ratios are high at the olfactory bulb and the striatal receptors and low at the myocardial receptors. This parameter represents an important discriminatory property of trihexyphenidyl enantiomers. The eudismic ratio of 83 found for the antagonism of ACh inhibition of adenylyl cyclase in myocardium is close to the value of 68 previously obtained for the antagonism of M_2-mediated negative inotropic effects in guinea-pig atrium (Lambrecht *et al.*, 1988) and with the values of 69 and 40 reported in radioligand binding assays of the M_2 receptor (Dorje *et al.*, 1991; Waelbroeck *et al.*, 1992). Thus, the sensitivity of the ACh inhibition of myocardial adenylyl cylcase to the trihexyphenidyl enantiomers is consistent with the involvement of the M_2 receptor subtype. On the other hand, eudismic ratios as high as those found in the present study for the antagonism of ACh effects in the olfactory bulb and striatum have been previously observed at the ileal and glandular M_3 receptors and at the striatal M_4 receptor (Lambrecht *et al.*, 1989; Waelbroeck *et al.*, 1992). Collectively, the antagonistic activity of trihexyphenidyl enantiomers clearly discriminates the olfactory bulb stimulatory receptor and the striatal inhibitory receptor from the putative cardiac M_2 inhibitory receptor.

On the basis of pharmacological data and in light of the distribution of the different molecular forms of muscarinic receptor in the brain, it has been proposed that the striatal muscarinic receptor coupled to inhibition of adenylyl cyclase is pharmacologically equivalent to the m4 gene product (M_4) (McKinney *et al.*, 1989; Elhert *et al.*, 1989). Previous studies have shown that the muscarinic stimulation of adenylyl cyclase in the olfactory bulb and the muscarinic inhibition of the enzyme in the striatum show similar sensitivities to a number of antagonists and have led to the hypothesis that a similar receptor subtype mediates the two responses (Olianas & Onali, 1991). The present finding that both the olfactory bulb and striatal receptors are blocked by (R)- and (S)-trihexyphenidyl with a similar potency provides support for the same classification being given to these receptors, i.e. M_4.

In conclusion, the present study shows that the muscarinic stimulation of adenylyl cyclase in rat olfactory bulb and the muscarinic inhibitions of the enzyme in striatum and heart are stereospecifically antagonized by the enantiomers of trihexyphenidyl and that the differential sensitivity to these drugs can be useful for identifying the receptor subtypes mediating these functional responses.

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